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EXHIBIT B

IEM101, a naturally attenuated *Vibrio cholerae* strain as carrier for genetically detoxified derivatives of cholera toxin.

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Running title: CT mutants delivery by attenuated *Vibrio cholerae*

ABSTRACT

Two mutants of cholera toxin (CTS106 containing a Pro106→Ser substitution and CTK63 containing a Ser63→Lys substitution) with greatly reduced or no toxicity respectively, were expressed in the naturally attenuated IEM101 *Vibrio cholerae* strain (El Tor, Ogawa) which does not express cholera toxin (CT). Expression was driven by the natural promoter of CT, or by a promoter known to induce strong *in vivo* expression such as *nir*B. In the rabbit ileal loop assay where 10^4 wild type bacteria were sufficient to induce fluid accumulation, 10^9 IEM101 expressing CTS106 bacteria were needed to induce some fluid accumulation, while IEM101 expressing CTK63 was inactive, even when 10^{10} cells were used. When used to immunize mice intranasally, all bacteria induced vibriocidal antibodies, however, anti-CT antibodies were not induced by bacteria expressing low levels of CTK63 under the control of the *ct* promoter. Anti-CT antibodies were successfully induced by bacteria expressing high levels of CTK63 under the control of the *nir*B promoter, or by bacteria expressing low levels of CTS106. These data show that antibodies against cholera toxin can be induced *in vivo* by high level expression of a non toxic mutant, or by using a mutant with residual ADP- ribosyltransferase activity.

In conclusion, we have shown that IEM101, a naturally attenuated *Vibrio* strain known to be safe and immunogenic in humans, can be engineered to express immunogenic levels of CTK63, and may represent an ideal candidate for vaccination against cholera.

1. Introduction

Endemic and epidemic cholera has been and remains a major health problem. It is estimated that more than 120,000 people die each year from cholera [1]. The disease is caused by the infection of *Vibrio cholerae*, a non-invasive enteropathogen that colonizes the mucosa of the small bowel and releases cholera toxin (CT), which stimulates the secretion of water and electrolytes [2]. CT has a typical A-B structure [3]. The A subunit is an enzyme with ADP-ribosyltransferase activity [4–6] responsible for the toxicity of the molecule, while the B subunit is non toxic and contains the GM1 ganglioside receptor-binding site [7,8]. The A subunit ADP-ribosylates the α subunit of G_s, a GTP-binding protein which controls the activity of adenylate cyclase [9–11]. Cholera is a disease that could be prevented by immunization with efficacious vaccines, since primary infection stimulates protective immunity against reinfection [12]. However, a vaccine which is entirely satisfactory for widespread use is not yet available, and even if important discoveries in the pathogenesis of *Vibrio* have been achieved in the last few years [13–16], the picture of the pathogenesis of the disease is still incomplete. A parenteral vaccine against cholera, composed of killed bacterial cells, is not recommended because of the side effects and the short-term protection induced [17,18].

Since *V. cholerae* colonizes the gastrointestinal tract, it is desirable to stimulate a mucosal immunity [19,20] able to block the bacterial adherence, opsonize or kill the bacteria and neutralize the toxin. This would render *V. cholerae* unable to persist and the toxin unable to exert its toxic effect. Two different strategies have been used to develop a mucosal (oral) vaccine against cholera. The first approach is based on the combination of killed whole *V. cholerae* 01 with the non toxic B subunit of CT (WC/rBS) [21–23]. The second approach is based on a live-attenuated *V. cholerae* strain (CVD 103-HgR), where the gene coding for the A subunit of CT has been deleted and thus expresses only the B oligomer [24–26]. The enzymatically active A subunit of CT has been excluded from these vaccine preparations because it is responsible for the toxicity, and also because

it was reported to induce insignificant titers of toxin neutralizing antibodies. The view that the A subunit is not useful in vaccines has been recently challenged by the results obtained with a number of non toxic derivatives of cholera toxin [27–30], showing that the holotoxin is more immunogenic than the B subunit and induces toxin neutralizing antibodies recognizing only the A subunit [31]. These results suggested that anti-A toxin neutralizing antibodies can be induced by using non toxic mutants of CT, and that the presence of the A subunit could be an useful addition to a cholera vaccine.

Here we propose a novel vaccine against cholera consisting of an attenuated *V. cholerae* strain producing a non toxic derivative of CT. The strain used is a naturally attenuated *V. cholerae* O1, El Tor, Ogawa strain, named IEM101, isolated in China and which does not contain the CTX genetic element [32]. IEM101 has been tested for safety and immunogenicity in humans. The results of this clinical trial showed that the strain is safe, able to colonize the intestinal mucosa, and to induce a strong immune response in terms of IgA, IgG and IgM and vibriocidal antibodies [32]. In the present study, IEM101 has been engineered to express two mutants of CT with no or very low toxic activity and used to study the toxicity and immunogenicity of the two CT mutants expressed *in vivo* under the control of different promoters. The mutants used are CTK63, which contains a site-directed substitution of the serine in position 63 of the A subunit of CT to lysine and which is devoid of any enzymatic activity and toxicity and CTS106, containing a substitution of the proline in position 106 to serine, which maintains residual levels of toxicity. Both mutants had been previously purified and successfully used for intranasal immunization of mice, in a study that showed that the presence of a residual enzymatic activity resulted in higher immunogenicity [33].

2. Materials and methods

2.1. Strains, media and growth conditions

Escherichia coli strain DH5α, or SY327λ*pir*, were used for cloning purposes.

E. coli SM10λ*pir* strain was used as donor strain for the conjugation. The attenuated *V. cholerae* strain El Tor Ogawa, named IEM101, was used as the recipient of plasmids containing the genes coding for the genetically detoxified CT derivatives, or for their chromosomal integration. Plasmids were transformed in *E. coli* using standard procedures [34], or electroporated in IEM101 as previously described [35]. *E. coli* recombinant strains were grown in LB medium or LB agar plates containing 100 μg/ml of ampicillin. IEM101 was grown in LB medium, or LB agar plates with 0.75 μg/ml of polimixin B, 0.75 μg/ml of gentamicine, and 100 μg/ml ampicillin when required. The conjugation was performed by cross-streaking on LB agar plates the recombinant *E. coli* SM10λ*pir*, transformed with the CVD422 suicide vector (Amp^r, $Sucrose^s$) [36], as donor strain and IEM101 as recipient strain in a 1:5 ratio. *V. cholerae* transconjugants were selected for the first recombination event on LB agar with 100 μg/ml of ampicillin, 0.75 μg/ml of polimixin and 0.75 μg/ml of gentamicin. To select for the second recombination event, in which the suicide vector (CVD422, Amp^r, $Sucrose^s$) was deleted from the chromosome, a single colony was grown overnight at 28°C in a modified LB medium (without NaCl) containing 10% sucrose. Dilutions of the overnight culture containing sucrose resistant bacteria were plated on LB agar supplemented with 20% sheep blood to check the hemolytic activity. The non-hemolytic colonies were replica-plated on LB with ampicillin or LB.

Liquid cultures were performed in water-bath rotary shakers in 500 ml flasks containing 100 ml of LB for IEM101, IEM-*ct*K63 or IEM-*ct*S106 respectively, at the temperature of 30°C. Low-aeration conditions of growth for IEM-*nir*K63 were achieved by inoculation of 1 ml from an overnight

culture into a completely filled, tight-capped 50 ml Falcon tube, and subsequent incubation at 37°C without agitation. For the time course experiments, IEM-*nir*K63 was grown in low aeration conditions in completely filled, tight-capped 15 ml Falcon tubes at 37°C. 15 ml samples of cells and culture supernatants were collected at various time intervals. Bacterial count was performed by plating serial dilutions of bacterial cultures on LB agar.

2.2. CT mutants construction, purification and characterization

The 1.1 Kb *Xba*I-*Hind*III fragment, containing the coding region of the *ctx* A and B subunit starting from *Xba*I site, was used as template for the site-directed mutagenesis using the following oligonucleotides: 5'-GTTTCCACCAAGATTAGTTTG-3' for Ser63→Lys and 5'-GGCATACAGTAGCCATCCAGA-3' for Pro106→Ser substitutions as previously described [31]. The mutated *Xba*I-*Hind*III fragment and the upstream regulatory regions of wild type CT were subcloned into the pEMBL19 vector as later described, generating the two recombinant plasmids p*ct*K63 and p*ct*S106, respectively. The two plasmids were electroporated in IEM101 and the purification of CTK63 and CTS106 mutant proteins was performed as previously described [31]. Both mutant molecules were analyzed for their ability to ADP-ribosylate polyarginine as described by Lai *et al.* [37]. *In vitro* and *in vivo* toxicity of the mutant proteins were evaluated using Y1 cells [38] and the rabbit ileal loop assay [39], respectively. Briefly, for the Y1 cells assay two-fold dilution of CT, CTK63 or CTS106 (starting from 80 pg well for wild type CT) were added to wells containing $5x10^4$ Y1 cells, and after 48 h cells were observed for morphological changes; for the rabbit ileal loop assay, one-milliliter samples containing various amounts of CT or CT mutants were injected into the intestinal loops of the rabbit (New Zealand). After 18-20 h the liquid accumulated into each loop was collected, measured with a syringe and results expressed as ml/cm. The rabbit ileal loop assay was also used to evaluate the toxicity of the recombinant IEM101 strains by injecting into the loops different dilutions of the bacteria.

2.3. Plasmids construction

The mutated *K63* and *S106* genes were placed under the control of the *ct* promoter as follows: a 260 bp DNA fragment, containing the wild type *ct* promoter, the upstream regulatory regions and the coding sequences of *ctx* gene up to *Xba*I site, was amplified from pGEM-CT vector [31], using the following oligonucleotides:

5'-AACCGAATTCAAGGCTGTGGGTAGAAGTG-3' (forward) containing a *Eco*RI site and 5'-CAGGAGGTCTAGAATCTGCCCGATAT-3' (reverse), containing a *Xba*I site. The amplified 260 bp fragment was digested with *Eco*RI and *Xba*I, ligated with the 1.1 Kb *Xba*I-*Hind*III DNA fragments deriving from the site-directed mutagenesis and containing the Ser63→Lys or Pro106→Ser substitutions, and cloned into pEMBL19 [40] digested *Eco*RI-*Hind*III, generating p*ct*K63 and p*ct*S106, respectively.

The *K63* gene was placed under the control of the *nir*B promoter, generating p*nir*K63, as follows: the 1.2 Kb fragment was amplified from p*ct*K63 using the following oligonucleotides: 5'-GGCCAAGTTAACTTCTGTTAAACAAAGGGAGCATTAT-3' (forward) and 5'-AAGTTAACGTCGACAAGCTTCTTAATTTGCCATACTAATT-3'(reverse), both containing a *Hpa*I site. The amplified fragment was digested with *Hpa*I and cloned into p*nir*100 [41], containing the *nir*B promoter, digested with *Eco*RV.

The IEM101 recombinant vaccine strains were generated inserting, by homologous recombination, the mutated *ctx* genes under the control of the *ct* or *nirB* promoters within the *hemolysin* (*hly*) gene. The subcloning of the flanking regions of the *hly* gene upstream and downstream to the *ctK63*, *ctS106* and *nirK63* genes was achieved as follows: the p*hly*3 plasmid, containing the *hly* gene (isolated from a IEM101 strain DNA library) as 3.5 Kb *Eco*RI-*Pst*I fragment cloned in Bluescript KS (D. Guangcai and R. Rappuoli, unpublished data) was digested with *Nru*I and *Hind*III. Following digestion, two fragments of 5 Kb and 1.5 Kb, respectively, were generated. The 1.5 Kb

fragment contains the core region of the *hly* gene, whereas the 5 Kb fragment contains the vector sequences, 1.2 Kb of the 5' end and 0.8 Kb of the 3' end of the *hly* gene. p*ct*K63, p*ct*S106 and p*nir*K63 were digested with *Eco*RI, filled with Klenow fragment of DNA polymerase I and digested with *Hind*III. The 1.3 Kb *Eco*RI/blunt-ended-*Hind*III fragments were cloned into the 5 Kb *Nru*I-*Hind*III fragment of p*hly*3, generating p*hly-ct*K63, p*hly-ct*S106 and p*hly-nir*K63, respectively.

The CVD422 suicide plasmid (obtained from Victor Di Rita, Medical School, University of Michigan) was used for the integration of the mutated genes into the *Vibrio* chromosome [36]. The three recombinant plasmids p*hly-ct*K63, p*hly-nir*k63 and p*hly-ct*S106 were digested with *Sac*I and *Sal*I, and the 3.3 Kb fragments were cloned into CVD422 digested with *Sac*I and *Sal*I, generating pCVD*hly-ct*K63, pCVD*hly-nir*K63, and pCVD*hly-ct*S106, respectively. These plasmids were propagated into the permissive SY327λ*pir* strain and then used to transform the SM10λ*pir* strain for conjugation purposes.

2.4. Analysis of protein expression and localization

To obtain periplasmic fractions and supernatant samples, 15 ml of cultures were harvested and centrifuged. Bacterial pellet was resuspended in 300 µl of 25% sucrose, 50 mM TrisHCl pH 8 and incubated with 1 mg/ml of polimixin B for 1 h at room temperature. The samples were centrifuged and the soluble fraction, containing the periplasmic extract, was isolated. 200 µl of culture supernatant and 4 µl of periplasm were analyzed for CT mutants expression and localization by ELISA. The presence of A and B subunits in the periplasm and/or in the culture supernatant was verified by Western blot using an anti-CT rabbit polyclonal sera at a dilution of 1:300. In the second case, to 50 ml of culture supernatant were added 2.5 g/l of Sodium Metaphosphate, the pH adjusted to 4.5 and the mixture incubated for 2.5 h at room temperature. The sample was centrifuged and the precipitate resuspended in 100 µl of sodium-phosphate buffer 100 mM pH 8. 50 µl were loaded on

15% SDS-PAGE. The amount of periplasmic extract loaded on each of the polyacrilamide gel for the Western blot analysis is reported in the figure legends.

2.5. Immunization of mice

6-8 weeks old female C57BL/6 mice were immunized with 10^8 bacteria in 30 µl of saline solution by intranasal route without anaesthesia, on days 0, 28, 42, 56. Animals were bled on days 27, 41, 55 and bled out on day 70. Further, at day 70, bile was recovered, nasal washes were performed by repeated flushing and aspiration of 1 ml of PBS, 0.1% BSA.

2.6. ELISA

The amount of CT mutants present in the culture supernatant or in the periplasm of the recombinant IEM101 strains was measured by GM1 capture ELISA. Each well of a 96-well plate (Greiner GmbH, Kremsmunster, Austria) was coated with 150 ng of the GM1 ganglioside (Sigma) (100 µl/well) by 2 h incubation at 37°C. Wells were then washed three times with PBS, 0.05% Tween-20 (PBT) and saturated with 1% BSA in PBS for 1 h at 37°C. 4 µl of periplasm in 200 µl of PBS, and 200 µl of supernatant were added in the first well and then serially diluted. Plates were incubated overnight at 4°C, and washed again with PBT. Wells were then incubated with 100 µl/well of a 1:20000 dilution of a polyclonal rabbit antisera for 2 h at 37°C, washed with PBT and incubated for 2 h at 37°C with 100 µl of a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antiserum. Plates were washed with PBT and developed with pNPP (Sigma) and A_{405nm} was read. The amount of CT mutants present in each sample was calculated on the basis of the absorbance (A_{405nm}) of known amounts of purified CT. Results were expressed as nanograms of CT for 10^7 cells.

To determine the amount of anti-CT antibodies present in sera, bile and mucosal washes of immunized mice, the ELISA was performed as described above. Briefly, to each well were added 150 ng of GM1, wells were washed as described above. 50 ng of wild type CT were added to each well and plates were incubated overnight at 4°C then were washed and saturated as described before. 100 µl of a 1:50 dilution of sera or bile and 100 µl of undiluted nasal washes were added and serially diluted. Plates were incubated for 2 h at 37°C. For detection of total Ig, wells containing serum samples were incubated with 50 µl of 1:1000 horseradish peroxidase (HRP) conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and incubated for 2 h at 37°C. For detection of IgA, wells containing serum samples, mucosal washes or bile were incubated with 50 µl of a 1:1000 dilution of biotin conjugated goat anti-mouse IgA α chain specific (Sigma) for 2 h at 37°C and, after washes with PBT, 50 µl of 1:1000 dilution of HRP-conjugated streptavidin were added to each well and plates incubated for 1 h at 37°C. Antigen-bound antibodies were visualized by adding o-phenylenediamine (OPD) as substrate (Sigma). After 10 min the reaction was blocked by the addition of 12,5% H_2SO_4 and the absorbance was read at 490 nm. ELISA titers in the sera were determined arbitrarily as the reciprocal of the last dilution which gave a $OD_{490nm} \geq 0.3$ above the preimmune sera, whereas for mucosal washes and bile IgA titers were expressed as the reciprocal of the last dilution which gave a $OD_{490nm} \geq 0.2$ above the preimmune sera. The values were normalized using positive control sera in each plate.

2.7. Serum vibriocidal titer

Vibriocidal activity was measured in a microassay using 96-well plates (Costar). The immunized animal sera were two-fold serially diluted in PBS, and aliquot of 50 µl were incubated for 1 h at 37°C with 50 µl of a solution containing $1x10^8$ CFU/ml culture of *V. cholerae* IEM101, 20% rabbit serum as complement source in PBS. 150 µl of BHI (brain and heart infusion, DIFCO) were then

added to each well, and plates were further incubated for 1 h at 37°C; absorbance at 570 nm was then measured. Titers were calculated as dilution of the serum that gave 50% growth inhibition, as compared to preimmune sera diluted 1:25.

3. Results

3.1. Characterization of CTK63 and CTS106 mutant proteins produced by IEM101

The two plasmids p*ct*K63 and p*ct*S106 containing the *ctxK63* and *ctxS106* genes under the control of the *ct* promoter were constructed as described in Material and Methods and then introduced in IEM101 by electroporation. The two mutant proteins CTK63 and CTS106 were purified from the supernatant of the recombinant IEM101 strains using a chromatographic step on CM-Sepharose [31] and analyzed for their biochemical and functional properties. The two molecules were correctly assembled in the AB_5 structure, efficiently secreted into the supernatant of IEM101 and equally susceptible to trypsin treatment and stable to long-term storage (data not shown). The enzymatic activity, *in vitro* and *in vivo* toxicity of the two mutant molecules are reported in Figure 1. The minimal amount of protein necessary to obtain detectable levels of enzymatic activity, in ADP-ribosylation assay using polyarginine as substrate, was 100 ng for wild type CT and 50 μg for CTS106. The CTK63 mutant was devoid of enzymatic activity even when 100 μg (the maximum amount tested) were used (Figure 1, panel *a*). The minimal amount able to induce morphological changes on Y1 cells, was 20 pg for wild type CT and 300 ng of CTS106. CTK63 was unable to induce any toxicity on Y1 cells, even when 6 μg (the maximum amount tested) were used in this assay (Figure 1, panel *b*). Finally, we tested the two mutant molecules in the rabbit ileal loop, the assay which is considered to be the most reliable to evaluate *in vivo* toxicity. As shown in Figure 1, panel *c*, 10 ng of wild type CT were needed to induce fluid accumulation whereas, for the CTS106 mutant, a significant increase in fluid accumulation was detected only when up to 100 μg were used in the assay. The CTK63 did not induce any fluid accumulation even at 1 mg, the maximum amount used. These data indicate that the CTS106 maintains a residual level of toxic activity which is 10^3-10^4 fold lower than wild type *in vitro*, and 10^4 fold lower *in vivo*. The CTK63 resulted completely

devoid of enzymatic activity and toxicity in all assays used.

3.2. Evaluation of CTK63 expression by using different promoters

To test whether the level of expression of the mutant proteins may affect the *in vivo* immunogenicity, the gene coding for CTK63, was placed under the control of *nir*B, a promoter that we had previously shown to be strong in *V. cholerae* [41]. The constructs were then integrated into the bacterial chromosome to avoid the problems deriving from plasmid instability during *in vivo* expression. The genes coding for CTK63 (under the control of *ct* or *nir*B promoters) and CTS106 (under the control of the *ct* promoter) were placed on the chromosome of IEM101 by homologous recombination, using the *hemolysin* (*hly*) gene as insertional locus. Allelic exchange was performed using derivatives of the suicide vector CVD442 [36], which contains the *sac*B gene from *Bacillus subtilis* and allows counter-selection for plasmid loss upon growth in medium containing sucrose. Recombinant IEM101 strains carrying the mutated *ctx* genes in the chromosomal *hly* gene were selected by plating on LB agar containing 20% blood. The three recombinant (non-hemolytic) strains IEM-*ct*K63, IEM-*ct*S106 and IEM-*nir*K63 were grown overnight in aerobic conditions, at the temperature of 30°C for IEM-*ct*K63 and IEM-*ct*S106 (for the induction of the ToxR-regulated *ct* promoter [42]) and 37°C for IEM-*nir*K63. Culture supernatants were precipitated with Sodium Metaphosphate and analyzed by Western blot. The results reported in Figure 2 show that levels of CTK63 and CTS106 produced were identical when expression was regulated by the *ct* promoter, whereas the expression of CTK63 was higher when expression was regulated by the *nir*B promoter, even in aerobic conditions of growth. Furthermore, under the growth conditions used, most of the A subunit was processed into the A_1 and A_2 peptides. To evaluate the amount of CTK63 produced following induction of the *nir*B promoter, the two recombinant strains (IEM-*ct*K63 and IEM-*nir*K63) were grown under selected conditions: 30°C for IEM-*ct*K63 and 37°C in aerobic or anaerobic conditions for IEM-*nir*K63 [43].

Bacterial cells were collected every 1.5 h and analyzed for bacterial counts and for CTK63 production and localization by ELISA and Western blots. The growth curve was identical for the two strains IEM-*ct*K63 and IEM-*nir*K63 when grown under aerobic conditions, while the bacteria growing under low-aeration had a lower growth. Starting from the third hour of culture, the duplication time increased and the number of bacteria reached values that were 10 times lower than the bacteria grown in aerobic conditions (data not shown). Expression and localization of CTK63 at 3, 4.5 and 6 h of growth, were quantified by ELISA and results are reported in Figure 3, panel *a*. When IEM-*ct*K63 and IEM-*nir*K63 were grown in aerobic condition all the CTK63 produced was found in culture supernatant; IEM-*nir*K63 production was approximately two-fold greater than that of IEM-*ct*K63. Higher levels of CTK63 expression were obtained when the IEM-*nir*K63 strain was grown in low-aeration conditions, reaching the maximum at 4.5 h of growth. Furthermore, in this case, some of the CTK63 produced remained in the periplasm of IEM101, suggesting that secretion of the toxin could be a limiting step.

To verify whether the amount of CTK63 detected in the ELISA assay was related to the entire holotoxin, culture supernatants from IEM-*nir*K63 corresponding to 3, 4.5 and 6 h of growth, were analyzed by Western blot. As shown in Figure 3 panel *b*, the supernatant contains both A and B subunits of CTK63. In this case the A subunit was not nicked into the A1 and A2 peptides even after 6 hour of growth, indicating that longer time of growth is necessary for the cleavage of the A subunit of CT by *V. cholerae* hemagglutinin/protease [44].

3.3. In vivo toxicity of recombinant strains

It has been shown that IEM101 is unable to induce fluid accumulation in the rabbit ileal loop assay [32]. To determine whether IEM101 expressing the mutated toxin was still

unable to induce any fluid accumulation *in vivo*, the three recombinant strains were tested in the rabbit ileal loop assay, that is believed to be the most reliable test to predict the *in vivo* toxicity of CT. As positive control wild type *V. cholerae* O395 strain was used. While the control strain induced fluid accumulation already when 10^4 bacteria were injected into the loop, 10^9 IEM-*ct*S106 bacterial cells were needed to induce detectable fluid accumulation (Figure 4). In the case of IEM-*ct*K63 and IEM-*nir*K63, even 10^{10} bacterial cells were unable to induce any fluid accumulation. These data are in agreement with those related to the purified mutant proteins (Figure 1, panel *c*) and suggest that both IEM-*ct*K63 and IEM-*nir*K63 could be safe in humans if used as live vaccine strains.

3.4. Immune response following intranasal immunization with the vaccine strains

The immune response induced by the recombinant IEM101 strains was evaluated in a mouse model of intranasal immunization. For this purpose, non-anesthetized C57BL/6 mice were immunized intranasally with 10^8 CFU of IEM101, IEM-*ct*K63 or IEM-*nir*K63. The presence of mutant toxins in the total cell extract of bacterial cells used for immunization was verified by Western blot (data not shown). Anti-vibriocidal titers and serum anti-CT Ig and IgA antibody titers were determined after each immunization. As shown in Figure 5, vibriocidal titers were detectable already after a single immunization and were identical in all groups of mice. Ig anti-CT antibodies were detectable only in sera of mice immunized with IEM-*nir*K63 or IEM-*ct*S106, while they were never detected in mice immunized with IEM-*ct*K63, or with IEM101 (Figure 6). IEM-*nir*K63 required at least two immunizations to induce an anti-CT response, whereas the response induced by IEM-*ct*S106 was detectable already after one immunization. A similar pattern was observed for anti-CT serum IgA antibodies that were detected only in mice immunized with IEM-*nir*K63 and IEM-*ct*S106 after three immunizations. The serum IgA titers induced by the two strains were comparable

(Figure 7, panel *a*). Anti-CT IgA were detected also in nasal washes and bile of mice immunized with IEM-*nir*K63 and IEM-*ct*S106, but not in mice immunized with IEM-*ct*K63 (Figure 7, panel *b* and *c*).

4. Discussion

IEM101, a naturally attenuated *V. cholerae* ElTor strain isolated in China that is safe and immunogenic in humans [32], could represent an ideal starting-point in the development of a new vaccine against cholera. Immunization with IEM101 elicited high titers of anti-LPS antibodies [32]. However, it is known that anti-toxin antibodies have a synergistic effect with anti-LPS antibodies in inducing protection against challenge with live *V. cholerae* [45]. In a field trial performed in Bangladesh, during the first six months of the trial, protection against cholera was higher (85%) in people vaccinated with the oral whole cell vaccine containing the purified B subunit and killed bacterial cells, than in people vaccinated with killed bacterial cells alone (58%) [46–48]. It has been also shown that the CT holotoxin is more immunogenic than the B subunit [33,49]. Overall, these data support the hypothesis that delivery of non toxic CT derivatives by live-attenuated *Vibrio* strains may increase the protection induced by the cholera vaccines. We have thus expressed two genetically detoxified derivatives of CT into IEM101. To avoid the loss of the plasmids carrying the mutated genes during bacterial replication *in vitro* and *in vivo*, the mutated genes were stably inserted on the chromosome of IEM101 and the activity of two different promoters, *ct* and *nirB* for *in vitro* expression were compared. The highest level of CTK63 production was obtained when the expression was driven by the *nir*B promoter, showing that *nirB* promoter is functional in IEM101. Immunogenicity of the recombinant vaccine strains was evaluated in a mouse model of intranasal immunization. We have previously reported that the intranasal route of immunization can be successfully used for the evaluation of the immune response induced by recombinant *Vibrio* strains [41]. Even if *Vibrio* is unable to replicate in the mouse, we have shown a persistence of the strain in the respiratory tract at least for 24 h after inoculation, and that the *Vibrio* strain must be alive in order to deliver heterologous

antigens to the host immune system [41]. In this paper we have shown that mice immunizations with the recombinant IEM101 strains resulted in equivalent levels of bactericidal antibodies, and that significant differences were detected in the anti-toxin immune response. In fact, when expression was driven by the *ct* promoter, despite the identical amount of both the mutant proteins (CTS106 and CTK63), only immunization with IEM101 expressing CTS106 raised anti-CT systemic and mucosal Ig and IgA immune responses. No Ig or IgA anti-CT immune responses were detected in sera and mucosal washes of mice immunized with IEM101 expressing CTK63. Interestingly, when the CTK63 was expressed in IEM101 under the control of the *nirB* promoter, the systemic and mucosal immune responses induced were equivalent to those induced by CTS106 IEM101-delivered. The low immunogenicity of CTK63 compared to CTS106 is in agreement with the results of a previous study in which the purified proteins were used as immunogens [33]. On the basis of that study we concluded that the ADP-ribosylation activity is important for the immunogenicity of CT. However, the data reported here support the hypothesis that the immunological effect induced by a CT mutant devoid of any enzymatic activity is dose-dependent. In fact, an increase in the amount of CTK63 IEM101-delivered induces systemic and mucosal immune responses comparable to those induced by IEM101 expressing CTS106. We conclude that CTK63 represents a safe and immunogenic molecule and that IEM101 expressing CTK63 could represent an ideal candidate for vaccination against cholera.

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Figure legends

Figure 1. Enzymatic activity, *in vitro* and *in vivo* toxicity of wild type CT, CTS106 and CTK63. *a*) ADP-ribosylation activity using polyarginine as substrate; *b*) *In vitro* toxicity on Y1 cells: minimal amounts of wild type CT and CTS106 able to induce full toxicity on Y1 cells (20 pg for wild type CT, and 300 ng of CTS106); for CTK63 the amount reported is the maximum amount tested (6 μg). *c*) *In vivo* toxicity in the Rabbit Ileal Loop (RIL): fluid accumulation induced by the three molecules expressed as the ratio of the amount of fluid collected (in ml) and the length of each loop (in cm). The mutant proteins are indicated as follows: wild type CT (●), CTS106 (■), CTK63 (Δ).

Figure 2. CT mutants expression. Western blot of supernatants deriving from IEM-*ct*S106, IEM-*ct*K63 and IEM-*nir*K63 overnight growth (in aerobic conditions). The A subunit is nicked into A_1 and A_2 fragments, of which A_1 is clearly recognizable on the gel, whereas the A_2 is not visible under the conditions used. Purified CT (3 μg) was used as standard. Anti-CT polyclonal serum was used at a dilution of 1:300.

Figure 3. CTK63 expression by IEM101 under control of *ct* or *nirB* promoters during growth and its localization. *a*) Amount of CTK63 in periplasms and in culture supernatants, in samples collected during growth of IEM-*ct*K63 and IEM-*nir*K63, determined by GM1-capture ELISA and expressed as ng of CTK63 present in 10^7 bacterial cells. *b*) Western blot of 40 μl of periplasmic fractions deriving from about $2.5x10^{10}$ IEM-*nir*K63 bacterial cells after 3, 4.5 and 6 h of growth in low aeration conditions, probed with anti-CT rabbit polyclonal sera at a dilution of 1:300, showing the A and B subunits. Purified CT (3 μg) was used as standard.

Figure 4. *In vivo* toxicity of recombinant IEM- *ct*K63, IEM- *ct*S106 and IEM-*nir*K63 strains in the Rabbit Ileal Loop assay. Fluid accumulation induced by 10^2, 10^4 and 10^6 CFU of wild type O395 *V. cholerae* strain (□); 10^2, 10^4, 10^6, 10^7, 10^8, 10^9 and 10^{10} CFU of IEM-*ct*S106 (■); 10^{10} CFU of IEM-*ct*K63 (○),IEM-*nir*K63 (◆), or IEM101 (Δ). Results are expressed as the ratio of the amount of fluid collected (in ml) and the length of each loop (in cm).

Figure 5. Serum vibriocidal activities. Serum vibriocidal titers in C57BL/6 mice after four immunizations with 10^8 bacteria. Titers were calculated by using dilution of pooled sera of eight mice per group. B1, B2, B3 and B4: sera after one, two, three or four immunizations.

Figure 6. Serum Ig anti-CT response. Ig immune response against CT in sera of eight mice, C57BL/6 strain, after receiving intranasally one to four doses of 10^8 bacteria. Results are shown as mean of anti-CT Ig titers, and error bars indicate the standard deviation from the mean titer. B1, B2, B3 and B4: sera after one, two, three or four immunizations.

Figure 7. Anti-CT serum and mucosal IgA responses. (*a*) IgA immune response in sera of mice immunized one to four times with 10^8 bacteria. Results are expressed as mean titers and error bars indicate the standard deviation from the mean titer. B1, B2, B3 and B4: sera after one, two, three or four immunizations.

IgA immune response in bile (*b*) and nasal washes (*c*) of mice immunized four times with 10^8 bacteria. Results are shown as individual titers.

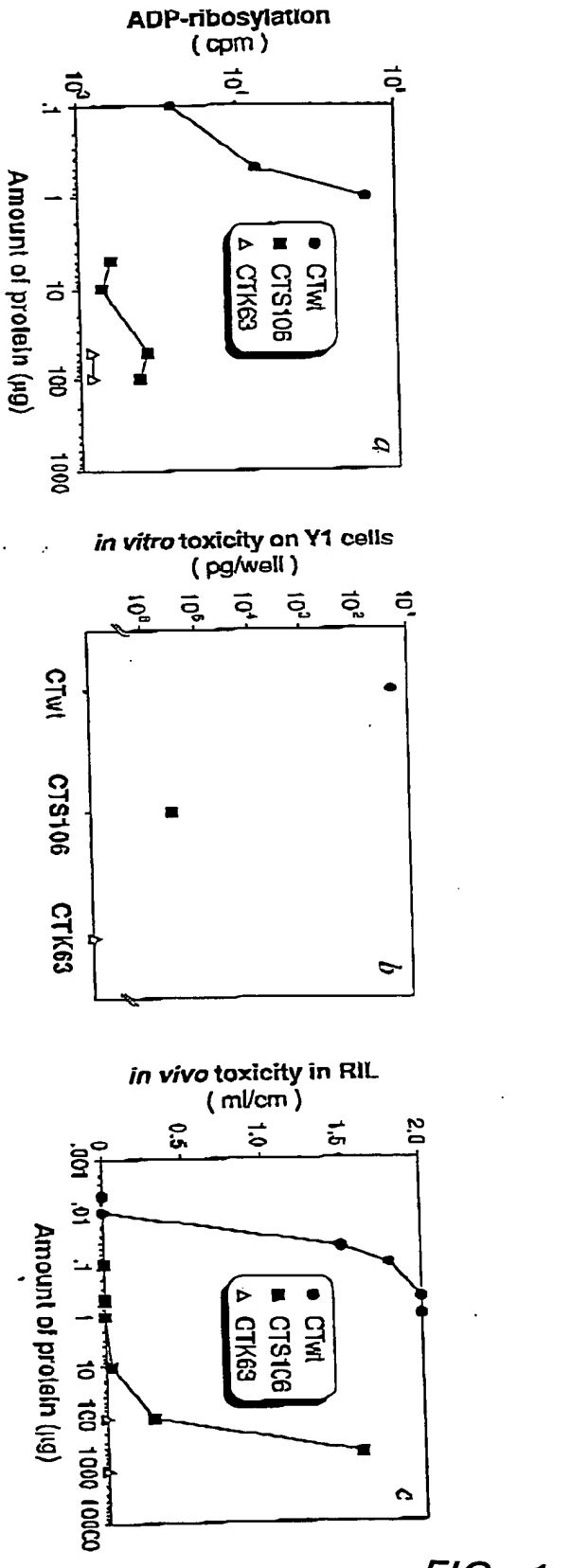
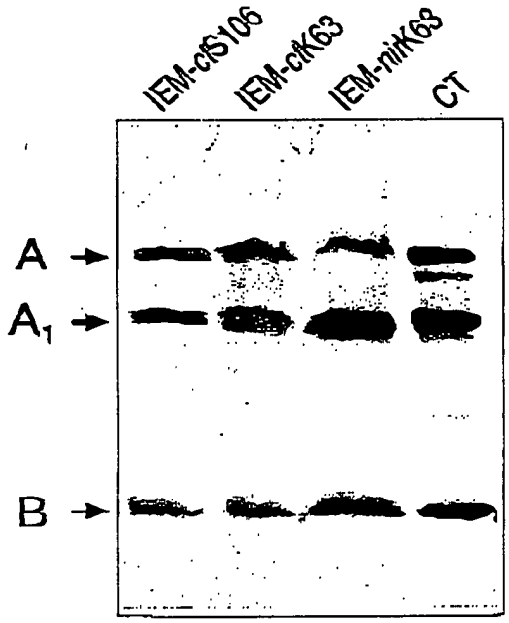
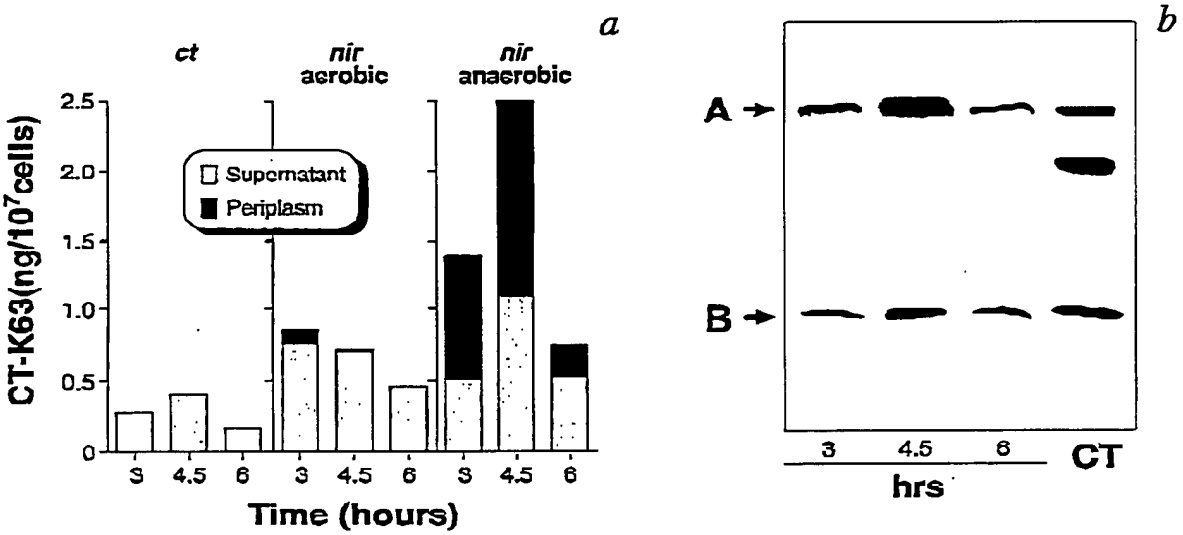
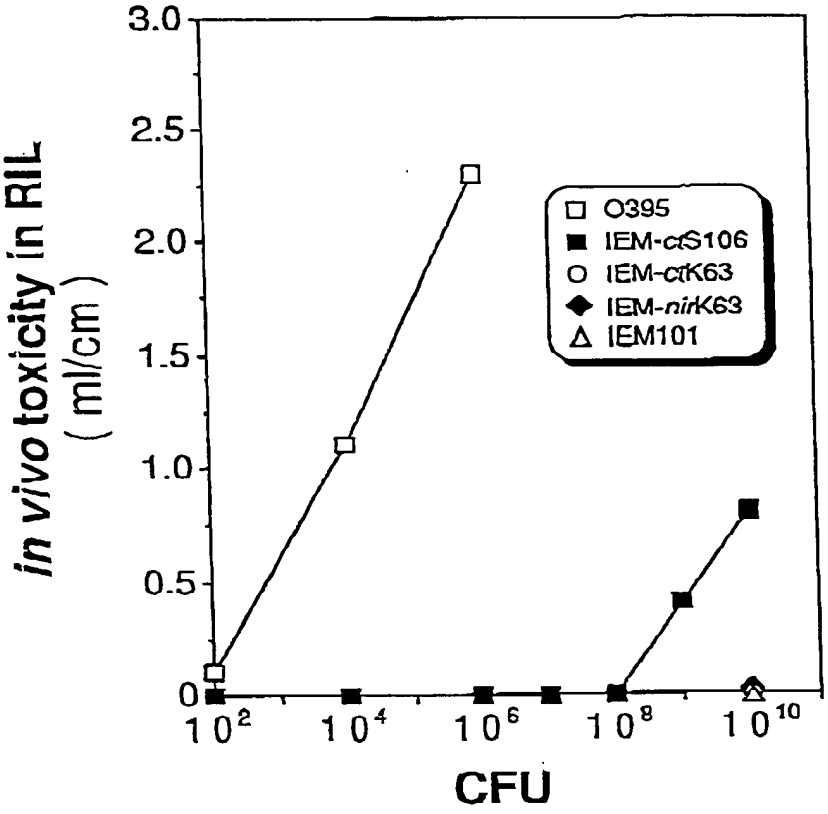
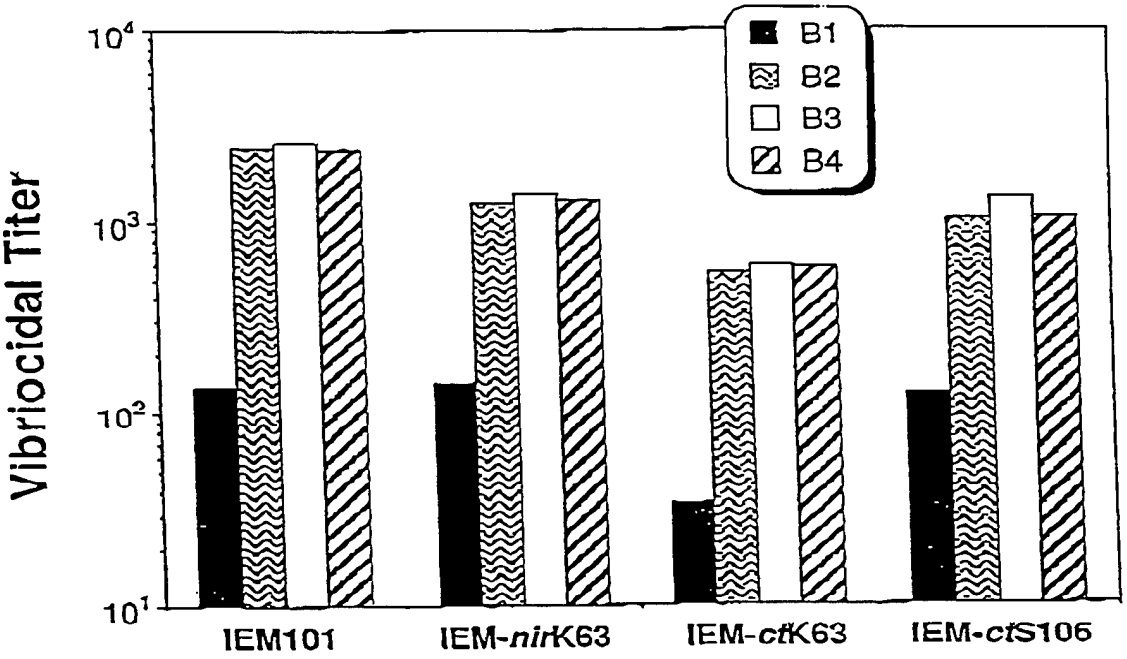

ADP-ribosylation
(cpm)
Amount of protein (µg)
CTwt
CTS106
CTK63
a
in vitro toxicity on Y1 cells
(pg/well)
CTwt
CTS106
CTK63
b
in vivo toxicity in RIL
(ml/cm)
Amount of protein (µg)
CTwt
CTS1C6
CTK63
c

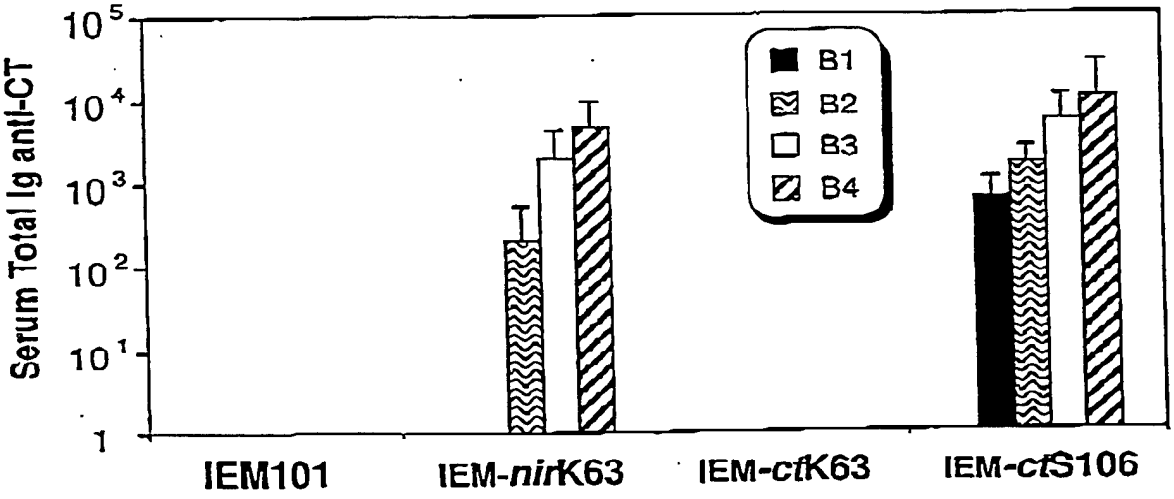
FIG. 1

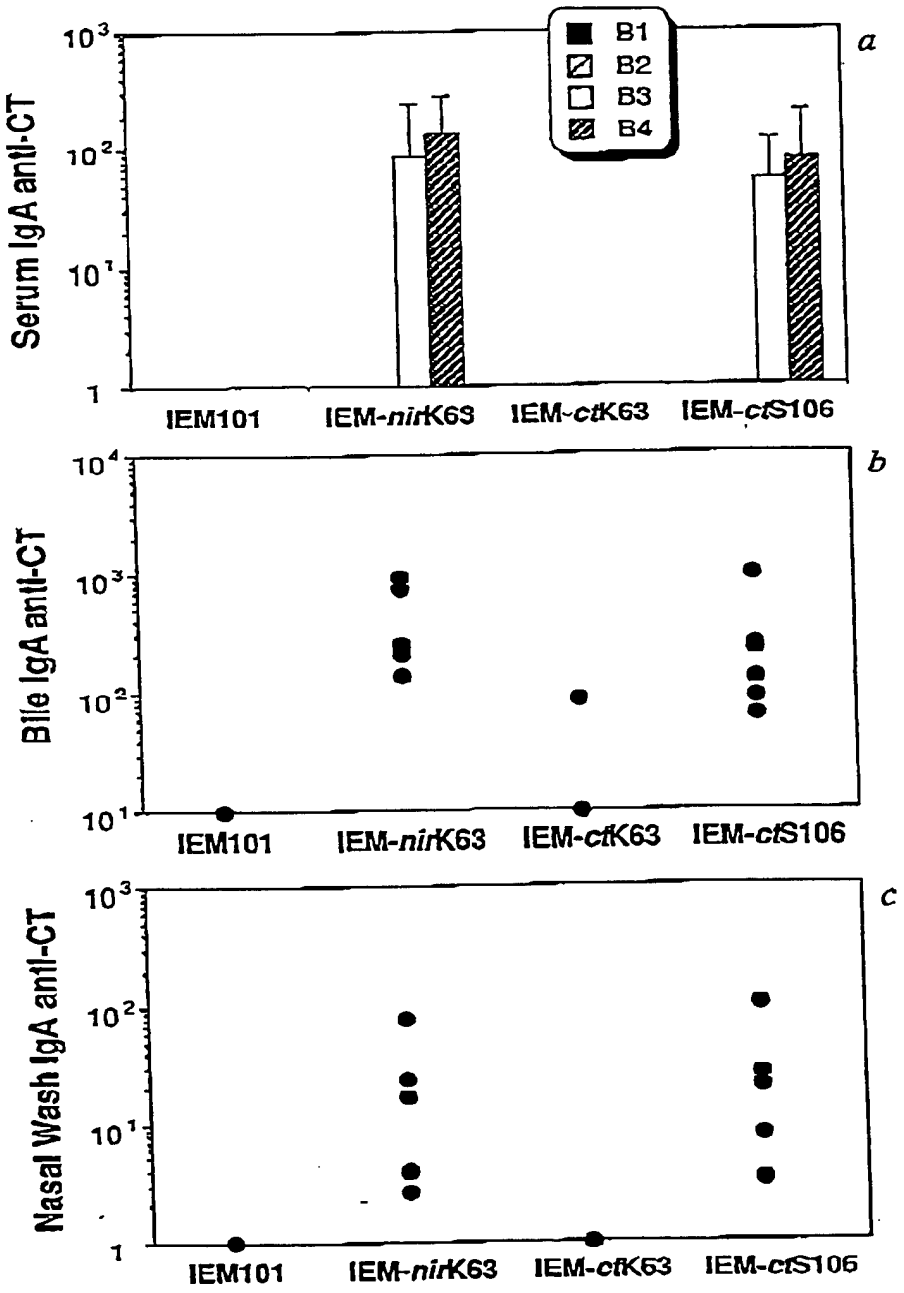

IEM-cfS106
IEM-cfK63
IEM-nifK63
CT
A
A1
B


a
ct
nir aerobic
nir anaerobic
CT-K63(ng/10^7cells)
2.5
2.0
1.5
1.0
0.5
0
Supernatant
Periplasm
3
4.5
6
Time (hours)
b
A
B
3
4.5
6
CT
hrs


3.0
2.5
2.0
1.5
1.0
0.5
0
in vivo toxicity in RIL
(ml/cm)
O395
IEM-ctS106
IEM-ctK63
IEM-nirK63
IEM101
10 2
10 4
10 6
10 8
10 10
CFU


Vibriocidal Titer
10^4
10^3
10^2
10^1
B1
B2
B3
B4
IEM101
IEM-*nir*K63
IEM-*ctk*K63
IEM-*ctS*106


Serum Total Ig anti-CT
10^5
10^4
10^3
10^2
10^1
1
B1
B2
B3
B4
IEM101
IEM-*nir*K63
IEM-*ct*K63
IEM-*ct*S106


a
Serum IgA anti-CT
10^3
10^2
10^1
1
B1
B2
B3
B4
IEM101
IEM-nirK63
IEM-ctK63
IEM-ctS106
b
Bile IgA anti-CT
10^4
10^3
10^2
10^1
IEM101
IEM-nirK63
IEM-ctK63
IEM-ctS106
c
Nasal Wash IgA anti-CT
10^3
10^2
10^1
1
IEM101
IEM-nirK63
IEM-ctK63
IEM-ctS106